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Figure 4A-4D. Factor addition influences the appearance of the nonadherent cell (NAC) type. Hoffman modulation contrast photographs were taken of cultures treated with various factors. Cultures were grown in 5% FBS for five days until a confluent monolayer was obtained; factors were then added for an additional 48 hours and the cultures were photographed. NACs were observed in all conditions. Panel A shows the control culture grown in FBS; panel B, culture treated with DCE (1  $\mu$ M Dexamethasone, 100 ng/ml Cholera toxin, 10 ng/ml EGF); Panel C, HGF (10 ng/ml), and Panel D, TGF $\beta$ 1 (10 ng/ml). Arrow 1 in Panel A indicates the adherent and confluent monolayer and the Arrow 2 points to a pair of rounded loosely or non-adherent cells. The HGF and TGF $\beta$ 1 treated cultures also contained semi- and non-adherent cells. However, the pharmacological cocktail DCE induced, on average, at least 8-fold more NACs than all other conditions tried. BrdU pulsing experiments showed strong proliferation and a confluent monolayer even after 48 hours of DCE exposure, indicating perhaps asymmetric division as opposed to simple loss of cell adherence. The inset in Panel B illustrates the morphology and granularity of NACs.

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Figure 5A-5G. Multiple hormone-containing cell types are detected in the NAC population. NACs were collected from DCE-stimulated monolayer cultures and analyzed immunocytochemically for endocrine marker expression. Cells expressing insulin (A, C), PDX-1 (D), glucagon (E), somatostatin (F), and pancreatic polypeptide (G) were all present in the NAC population. Markers were visualized with FITC or Cy3 immunofluorescence and the nuclei counterstained with DAPI (C-G). Panel A shows a 10X objective field magnification of insulin staining. Heterogeneous signal strength was observed; shown here are one brightly stained cell and many dimly stained cells along with negative cells. Panel B shows staining with normal preimmune serum. Note that the dim cells in A are significantly above background, yet contain much less insulin than the bright cell observed. Panel C shows higher magnification (20X objective) of another insulin staining (Cy3) showing dim and negative cells. In this field and others approximately 40-50% of the cells test insulin-positive. Panels D, E, F, and G show PDX-1, glucagon, somatostatin and pancreatic polypeptide staining, respectively (60X objective). Arrows indicate DAPI-stained, hormone-negative cells.

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**Figure 6A-6D.** Single cell PCR (SC-PCR) analysis of PDX-1, insulin and glucagon expression in NACs. Forty cells were selected from a random population of NACs and processed for cDNA as described in the Methods section. These cDNAs were then analyzed for insulin (B), glucagon (C), and PDX-1 (D) message. Panel A shows the ethidium bromide staining of the cDNA on a 1.2% agarose gel. The bulk of cDNA product fell within the targeted 500-1000 bp range. Panel B shows that there is variation in the amount of insulin message per cell, with some cells giving much stronger signal than others. 15/40 (37%) of the cells tested positive for insulin mRNA. Of these, one was also positive for glucagon, and both messages were relatively weak compared to the other cells that expressed insulin only. Panel C shows that 2/40 (5%) of the cells contained glucagon message, a result that correlates well with the immunocytochemistry data. Panel D shows that many of the picked cells contained PDX-1 mRNA. Note that a significant fraction of cells express PDX-1 mRNA only, with no insulin or glucagon.

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**Figure 8A-8B.** Glucose-induced calcium currents in NACs. Glucose induces an inward calcium current in the NAC population. Changes in intracellular calcium at the single cell level were monitored using Fluo-3 and confocal microscopy. A, In this representative experiment ( $n > 10$ ) approximately one-third of the population initiated a robust calcium influx in response to glucose administration, and 58% of the cells showed no response to glucose. In every experiment, approximately 6-10% of the cells begin with a high intracellular calcium content that decreases with time; these were judged to be dying cells. 80 cells were analyzed in this experiment. B, The reversibility of the induced calcium current is demonstrated. In this representative experiment ( $n > 6$ ), the glucose-stimulated calcium current could be washed out with Krebs Ringer Phosphate (KRP) solution. A second calcium current could then be stimulated by readministration of 17 mM glucose. Washout of the glucose followed by tolbutamide stimulation, a SUR-linked potassium channel blocker, also stimulated a calcium current, as expected. Arrows indicate times of administration. A total of 123 cells were analyzed in this experiment. 7-13 % of the cells gave rise to calcium currents in response to the stimulus (shown in red) whereas 45-65 % of the cells showed no response to any of the stimuli

BB (shown in blue). The remaining 35% of cells exhibited varying amplitudes and kinetics in response to challenge, indicating a complex population.

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**Figure 41.** Pancreatic duct spheres grown in expansion medium. P14 rat pancreas was collected and digested, and ducts were collected by Percoll gradient centrifugation of the tissue suspension. Dissociated duct cells were seeded and cultured in expansion medium.

**Figure 42.** P14 duct spheres cultured in three different media.

BB **Figure 43.** Partial differentiation of duct spheres in vitro. Duct spheres were seeded into 96-well plates containing HTB9 matrix in differentiation medium. Following plating, cells migrated out of the spheres and formed a monolayer. Antibody staining using anti-nestin and anti-Glut2 antibodies reveal expression of both markers. However, Glut2 positive cells are in clusters and are excluded from nestin positive cells.

**Figure 44.** Partial differentiation of duct spheres in vitro. Duct spheres were seeded into 96-well plates containing HTB9 matrix in differentiation medium. Following plating, cells migrated out of the spheres and formed a monolayer. Antibody staining using anti-Pdx1 and anti-glucagon antibodies reveal expression of both markers. However, we did not observe expression of both markers within a single cell.

**Figure 45.** Partial differentiation of duct spheres in vitro. Duct spheres were seeded into 96-well plates containing HTB9 matrix in differentiation medium. Following plating, cells migrated out of the spheres and formed a monolayer. Antibody staining using anti-Pdx1 and anti-insulin antibodies reveal expression of both markers. Expression of Pdx1 and insulin are tightly associated with approximately 16% of Pdx1+ cells co-expressing insulin.

**Figure 46A-46B.** Graphs representing insulin and Glut2 protein expression under both expansion and differentiation conditions.

**Figure 47.** Expression of nestin in duct spheres in the growth state. Undifferentiated duct spheres were grown, dissociated, and put onto cytopsin slides. Antibody staining with anti-nestin antibodies revealed that a greater proportion of cells express nestin under growth conditions than under differentiation condition.

**Figure 48.** Table summarizing expression data for duct spheres in the growth state. Undifferentiated duct spheres were grown, dissociated and put onto cytopsin slides. Antibody staining with anti-nestin, anti-Glut2, anti-Pdx1, and anti-insulin antibodies was performed.

Figure 49-51. Micrographs of various hematopoietic cell sphere ("Hemesphere") cultures generated from E13 mouse fetal livers.

Figure 52-59. FACS analysis of hemisphere cultures generated from E13 mouse fetal livers.

Figure 60. Histological analysis of cells in hemisphere culture.

*The replacement paragraphs presented above incorporate changes as indicated by the marked-up versions below.*

Figure 3A-3G. The duct monolayer expresses multiple progenitor cell markers. Monolayers were stained for both insulin (A) and amylase (B). Panel C is a composite showing that some cells express both insulin and amylase. Two morphologically distinct cell types are present, those that are adherent and flat, and cells that are semi-adherent and round. Arrowheads denote\* rounded semi-adherent cells that may coexpress both insulin and amylase. Panels D and E show staining for glucagon and PYY, respectively, and Panel F is a composite showing that one of the glucagon-bright cells also expresses PYY. Panel G shows a composite of nuclear PDX-1 (Cy3) and cytoplasmic insulin (FITC) staining. Arrowheads indicate cells that express PDX-1 but not insulin or vice versa.

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